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Determination of Rotenone in Industrial and Municipal Wastewaters

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DETERMINATION OF ROTENONE IN INDUSTRIAL AND MUNICIPAL WASTEWATERS

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A method was developed for the determination of rotenone in wastewaters. The method development program consisted of a literature review; determination of extraction efficiency for each compound from water into methylene chloride; development of a deactivated silica gel cleanup procedure; and determination of a suitable high performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection.

The final method was applied to a relevant industrial wastewater to determine the precision and accuracy of the method. The wastewater was spiked with rotenone at levels of 5.5 µg/L and 110 µg/L. Recovery for rotenone at the 5.5 µg/L level was 85 ± 8 percent. Recovery at the 110 µg/L level was 88 ± 3 percent. The method detection limit (MDL) for rotenone in distilled water was 1.6 µg/L. The MDL in wastewaters may be higher due to interfering compounds.

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FOREWORD

Environmental measurements are required to determine the quality of ambient waters and the character of waste effluents. The Environmental Monitoring and Support Laboratory - Cincinnati, conducts research to:

- Develop and evaluate methods to measure the presence and concentration of physical, chemical, and radiological pollutants in water, wastewater, bottom sediments, and solid wastes.

- Investigate methods for the concentration, recovery, and identification of viruses, bacteria, and other microbiological organisms in water; and, to determine the responses of aquatic organisms to water quality.

- Develop and operate an Agency-wide quality assurance program to assure standardization and quality control of systems for monitoring water and wastewater.

- Develop and operate a computerized system for instrument automation leading to improved data collection, analysis, and quality control.

This report is one of a series that investigates the analytical behavior of selected pesticides and suggests a suitable test procedure for their measurement in wastewater. The method was modeled after existing EPA methods being specific yet as simplified as possible.

Robert L. Booth, Director
Environmental Monitoring and Support
Laboratory - Cincinnati

ABSTRACT

A method was developed for the determination of rotenone in wastewaters. The method development program consisted of a literature review; determination of extraction efficiency for each compound from water into methylene chloride; development of a deactivated silica gel cleanup procedure; and determination of a suitable high performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection.

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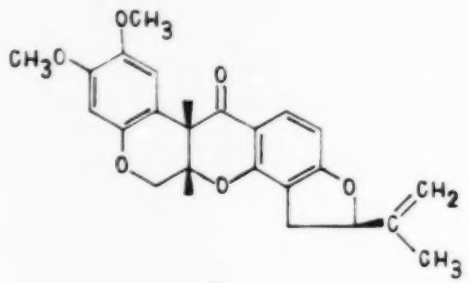
SECTION 1

INTRODUCTION

Rotenone (I) is a naturally occurring insecticide derived from derris root. It is used primarily to control insects on food crops, but is also used to eradicate undesirable fish species from lakes and streams.



I

The CAS registry number for rotenone is 83-79-4 and its IUPAC name is [2R(-2a, 6a α, 12aα,)]1,2,12,12a-tetrahydro-8,9-dimethoxy-2-(1-methylethenyl) [1] benzopyrano(3,4-V) furo[2,3-h] [1] benzopyran-6(6aH)-one. It has a melting point of 165-166°C and an oral LD50 in rats of 133 mg/Kg. Common synonyms for rotenone include "Derris" and "cube". Several papers have been published which describe the analysis of rotenone using HPLC (1-6). Most of the methods use reverse phase liquid chromatography with UV absorbance detection. A method using normal phase HPLC has also been reported (1). The detection wavelengths include 254 nm (4), 280 nm (2-5), and 294 nm (1,3,4,5). Methods for trace analysis using HPLC have been published for rotenone residues on crops (4,5,6), in animal feed and tissues (3). Cleanup procedures using silica gel have also been described (3,4,5,6).

Rotenone is stable in water at neutral pH and can be extracted from water with methylene chloride. Rotenone decomposes upon exposure to light and air; precautions should be taken to avoid excessive exposure of rotenone containing solutions to light and air (7). The selected approach to the determination of rotenone in water include separatory funnel extraction from water with methylene chloride; cleanup using silica gel chromatography; and analysis using HPLC with UV detection. Standard concentration techniques using Kuderna-Danish (K-D) equipment were used. The final method is included in Appendix A of this report.

SECTION 2

CONCLUSIONS

EXTRACTION AND CONCENTRATION

Rotenone can be extracted from water into methylene chloride with greater than 90 percent recovery using separatory funnel techniques. Use of K-D concentration equipment to perform extract concentrations did not significantly affect compound recoveries.

CLEANUP

Rotenone elutes from six percent deactivated silica gel with greater than 90 percent recovery. This was an effective cleanup procedure for a relevant wastewater sample.

CHROMATOGRAPHY

Two HPLC columns, Dupont Zorbax-Cyano (normal phase) and Spherisorb-ODS (reversed phase), were found to be acceptable for this application. The Dupont Zorbax-Cyano does not require an additional solvent exchange step and was used as the primary column. The Spherisorb-ODS column was designated as the alternate column.

VALIDATION STUDIES

Recoveries of rotenone from distilled water in the 5.5 to 1090 µg/L concentration range averaged greater than 85 percent. The analytical curve constructed from this data was linear. The MDL in distilled water was 1.6 µg/L. Recoveries of rotenone from a pesticide manufacturing wastewater at the 5.5 and 110 µg/L levels were 85 ± 8 and 88 ± 3 percent, respectively.

SECTION 3

EXPERIMENTAL

Studies were performed to determine if extractions with separatory funnels, cleanup by silica gel adsorption chromatography, concentration using K-D equipment, and analysis using HPLC with UV detection would be applicable technique for the determination of rotenone in water.

EXTRACTION AND CONCENTRATION

Extraction of rotenone from water was studied by using separatory funnel techniques. The distilled water was spiked with rotenone at the 10 µg/L and 100 µg/L levels. The sample was adjusted to pH 7 by addition of 1 N sodium hydroxide or 1 N sulfuric acid and extracted three times with 60 mL each of methylene chloride. These studies were done in duplicate. The extracts were dried by passing them through 10 cm of anhydrous granular sodium sulfate, concentrated to five mL and analyzed by HPLC. Column 1 was used for HPLC analyses.

CLEANUP

A 10-gram silica gel column (six percent deactivaated with water) was prepared as follows: 10 g silica gel was slurried with 50 mL of acetone containing 600 µL of reagent water, the slurry was transferred to a chromatographic column and the solvent was allowed to elute and was discarded. The column was rinsed with 100 mL of methylene chloride which was also discarded. The rotenone, 10 or 100 µg dissolved in one mL of methylene chloride, was added to the top of the column. The column was eluted with 50 mL portions of methylene chloride (F1), 6 percent acetone in methylene chloride (F2), 15 percent acetone in methylene chloride (F3), and 25 percent acetone in methylene chloride (F4). Each fraction was concentrated to five mL and analyzed by HPLC. Column 1 was used for HPLC analyses.

CHROMATOGRAPHY

Two HPLC columns were evaluated for the determination of rotenone: Dupont Zorbax-Cyano and Spheriorb-ODS.

VALIDATION STUDIES

The MDL for rotenone was determined by analyzing seven replicate distilled water samples spiked at the 5.5 µg/L concentration levels (8). The sample extracts were cleaned up using the silica gel cleanup procedure

prior to analysis. The amounts recovered were determined by external standard calibration and the MDLs were calculated from these data.

Distilled water was also spiked in duplicate at the 5.5, 22, 54, 270, and 1090 µg/L concentration levels and recoveries of the rotenone were determined as described earlier. An analytical curve was generated by plotting the amount spiked into the samples versus the amount recovered from the samples.

A relevant wastewater was used for wastewater validation studies. Seven replicates of the wastewater were analyzed to determine the background levels. The wastewater was spiked with rotenone at the 5.5 and 110 µg/L concentration levels, processed and analyzed. Seven replicate extractions were performed at each concentration level.

SECTION 4

RESULTS AND DISCUSSION

EXTRACTION AND CONCENTRATION

Rotenone was extracted from water with greater than 85 percent recovery using separatory funnel techniques. Recoveries of rotentone from water using separatoy funnels were 87 and 97 percent at the 10 µg/L level and 92 and 98 percent at the 100 µg/L level. These data are results of duplicate analyses.

CLEANUP

Rotenone eluted from six percent deactivated silica gel in the six percent acetone in methylene chloride fraction. Recoveries of 10 µg and 100 µg of rotenone were 98 and 93 percent, respectively.

CHROMATOGRAPHY

Both the Dupont Zorbax-Cyano and Spherisorb-ODS columns were satisfactory for the determination of rotenone. Use of the Dupont Zorbax-Cyano column requires one less solvent exchange step in the sample workup and was chosen as the primary column. The following conditions were used:

	Column 1
Column:	Dupont Zorbax-Cyano, 5 micron, 250 x 4.6 mm
Solvent:	30 percent methylene chloride, 70 percent hexane.
Flow:	1 mL/min.
Detector:	UV @254 nm
Injector Volume:	10 µL

	Column 2
Column:	spherisorb-ODS, 5 micron, 250 x 4.6 mm
Solvent:	60 percent acetonitrile, 40 percent water.
Flow:	1 mL/min.
Detector:	UV @254 nm
Injector Volume:	10 µL

Chromatograms obtained under these conditions are shown in Figures 1 and 2.

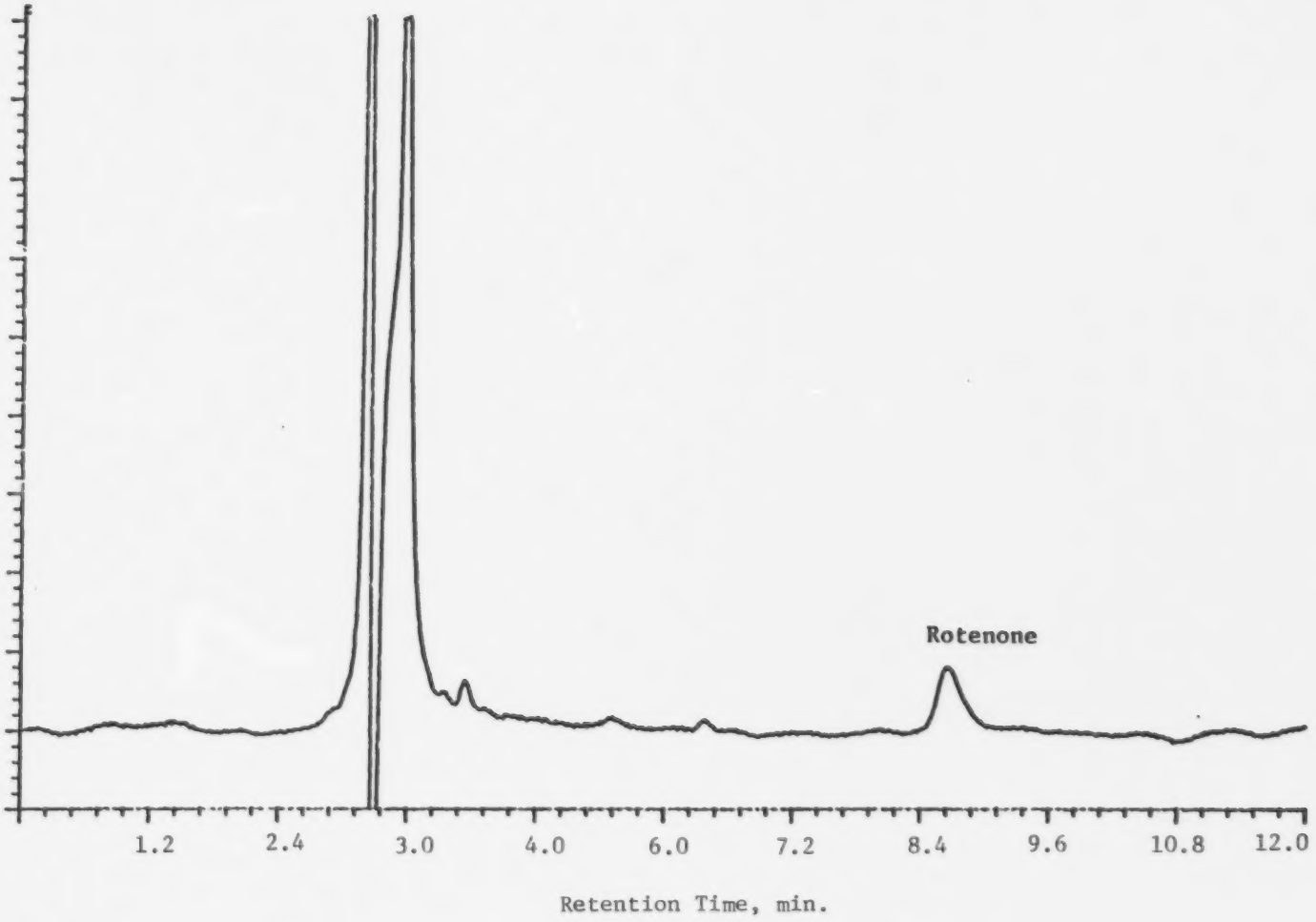


Figure 1. HPLC-UV Chromatogram of Standard Solution Representing 5 µg/L of Rotenone in Water (Column 1)

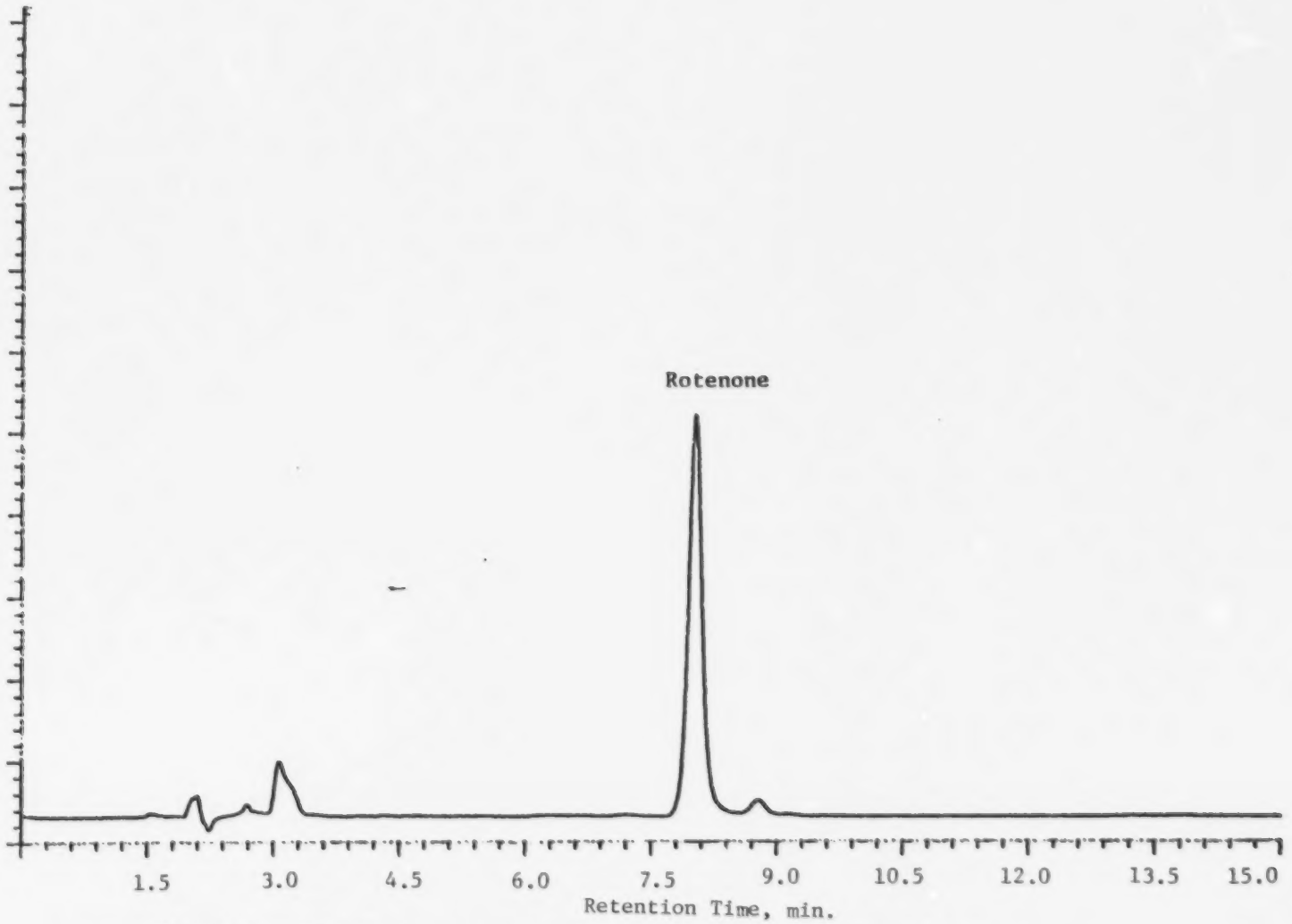


Figure 2. HPLC-UV Chromatogram of Standard Solution Representing 200 µg/L of Rotenone in Water (Column 2).

VALIDATION STUDIES

Recovery of rotenone from distilled water at the 5.5 µg/L level was 4.6 ± 0.5 µg/L. The MDL in distilled water was calculated to be 1.6 µg/L. Recoveries of rotenone from distilled water at the 5.5, 22, 54, 270, and 1090 µg/L levels were 4.6(17), 20(20), 45(4.4), 240(10), and 950(2.3) µg/L, respectively. These data were the averages of duplicate analyses. The percent relative range is given in parentheses. The resultant analytical curve is shown in Figure 3.

Recoveries of rotenone from a relevant wastewater at the 5.5 and 110 µg/L levels were 85 ± 8 percent and 88 ± 3 percent, respectively. These data were the averages of seven replicate analyses. Rotenone was not detected in the relevant wastewater.

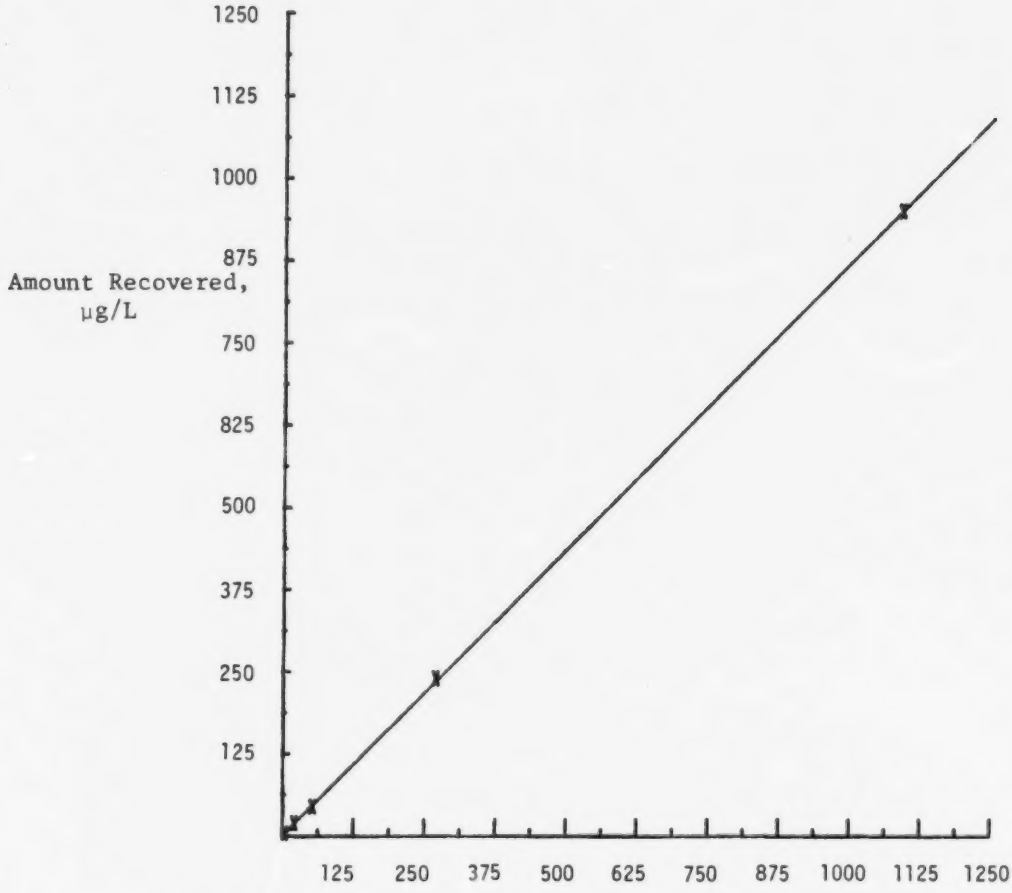


Figure 3. Analytical Curve for Rotenone

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DETERMINATION OF ROTENONE IN INDUSTRIAL AND MUNICIPAL WASTEWATERS BY LIQUID CHROMATOGRAPHY

METHOD 635

1. Scope and Application

1.1 This method covers the determination of rotenone pesticide. The following parameter can be determined by this method:

Parameters	CAS No.
Rotenone	83-79-4

1.2 This is a high performance liquid chromatographic (HPLC) method applicable to the determination of the compound listed above in municipal and industrial discharges as provided under 40 CFR 136.1. Any modification of this method beyond those expressly permitted shall be considered a major modification subject to application and approval of alternate test procedures under 40 CFR 136.4 and 136.5.

1.3 The method detection limit (MDL, defined in Section 14) for rotenone compound is listed in Table 1. The MDL for a specific wastewater may differ from those listed, depending upon the nature of interferences in the sample matrix.

1.4 This method is restricted to use by or under the supervision of analysts experienced in the use of liquid chromatography and in the interpretation of liquid chromatograms. Each analyst must demonstrate the ability to generate acceptable results with this

or muffle furnace at 400°C for 15-30 min. Some thermally

1.5 When this method is used to analyze unfamiliar samples for the compound above, compound identifications should be supported by at least one additional qualitative technique. This method describes analytical conditions for a second liquid chromatographic column that can be used to confirm measurements made with the primary column.

2. Summary of Method

2.1 A measured volume of sample, approximately 1 liter, is solvent extracted with methylene chloride using a separatory funnel. Liquid chromatographic conditions are described which permit the separation and measurement of the compounds in the extract by HPLC-UV.[1]

3. Interferences

3.1 Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware that lead to discrete artifacts or elevated baselines in liquid chromatograms. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5.

3.1.1 Glassware must be scrupulously cleaned.[2] Clean all glassware as soon as possible after use by thoroughly rinsing with the last solvent used in it. Follow by washing with hot water and detergent and thoroughly rinsing with tap and reagent water. Drain dry, and heat in an oven or muffle furnace at 400°C for 15-30 min. Some thermally

stable materials such as PCBs may not be eliminated by this treatment. Thorough rinsing with acetone and pesticide quality hexane may be substituted for the heating. After drying and cooling, seal and store glassware in a clean environment to prevent accumulation of dust or other contaminants. Store inverted or capped with aluminum foil.

3.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all-glass systems may be required.

3.2 Matrix interferences may be caused by contaminants that are coextracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the industrial complex or municipality being sampled. The cleanup procedure in Section 11 can be used to overcome these interferences, but unique samples may require additional cleanup approaches to achieve the MDL listed in Table 1.

4. Safety

4.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the

chemical analysis. Additional references to laboratory safety are available and have been identified[3-5] for the information of the analyst.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete or composite sampling.

5.1.1 Grab sample bottle - Amber borosilicate or flint glass, 1-liter or 1-quart volume, fitted with screw caps lined with Teflon. Aluminum foil may be substituted for Teflon if the sample is not corrosive. If amber bottles are not available, protect samples from light. The container and cap liner must be washed, rinsed with acetone or methylene chloride, and dried before use to minimize contamination.

5.1.2 Automatic sampler (optional) - Must incorporte glass sample containers for the collection of a minimum of 250 mL. Sample containers must be kept refrigerated at 4°C and protected from light during compositing. If the sampler uses a peristaltic pump, a minimum length of compressible silicone rubber tubing may be used. Before use, however, the compressible tubing should be thoroughly rinsed with methanol, followed by repeated rinsings with distilled water to minimize the potential for contamination of the sample. An integrating flow meter is required to collect flow proportional composites.

5.2 Glassware (All specifications are suggested. Catalog numbers are included for illustration only.)

5.2.1 Separatory funnel - 2000-mL, with Teflon stopcock.

5.2.2 Drying column - Chromatographic column 400 mm long x 10 mm ID with coarse frit.

5.2.3 Chromatographic column - 400 mm long x 19 mm ID with 250 mL reservoir at the top and Teflon stopcock (Kontes K-420290) or equivalent).

5.2.4 Concentrator tube, Kuderna-Danish - 25-mL, graduated (Kontes K-570050-2525 or equivalent). Calibration must be checked at the volumes employed in the test. A ground glass stopper is used to prevent evaporation of extracts.

5.2.5 Evaporative flask, Kuderna-Danish - 250-mL (Kontes K-570001-0250 or equivalent). Attach to concentrator tube with springs.

5.2.6 Snyder column, Kuderna-Danish - three-ball macro (Kontes K-503000-0121 or equivalent).

5.2.7 Snyder column, Kuderna-Danish - two-ball micro (Kontes K-569001-0219 or equivalent).

5.2.8 Vials - Amber glass, 5 to 10 mL capacity with Teflon lined screw-cap.

5.2.9 Volumetric flask - 10 mL.

5.2.10 Erlenmeyer flask - 250 mL.

5.2.11 Graduated cylinder - 1000 mL.

5.3 Boiling chips - approximately 10/40 mesh carborundum. Heat to 400°C for 4 hours or extract in a Soxhlet extractor with methylene chloride.

5.4 Water bath - Heated, capable of temperature control ($\pm 2^{o}C$). The bath should be used in a hood.

5.5 Balance - Analytical, capable of accurately weighing to the nearest 0.0001 g.

5.6 Liquid chromatograph - Analytical system complete with liquid chromatograph and all required accessories including syringes, analytical columns, detector and strip-chart recorder. A data system is recommended for measuring peak areas.

5.6.1 Pump - Isocratic pumping system, constant flow.

5.6.2 Column 1 - Normal-phase column, 5 micron Zorbax-CN, 250 x 4.6 mm or equivalent.

5.6.3 Column 2 - Reversed-phase column, 5 micron Spherisorb-ODS, 250 x 4.6 mm or equivalent.

5.6.4 Detector - Ultraviolet absorbance detector, 254 nm.

6. Reagents

6.1 Reagent water - Reagent water is defined as a water in which an interferent is not observed at the method detection limit of each parameter of interest.

6.2 Methylene chloride, methanol, acetonitrile, acetone, hexane, distilled-in-glass quality or equivalent.

6.3 Sodium sulfate (ACS) Granular, anhydrous; heated in a muffle furnace at 400°C overnight.

6.4 1N sulfuric acid.

6.5 1N sodium hydroxide.

6.6 Silica gel, Davison grade 923, 100-200 mesh, dried for 12 hours at 150°C.

6.7 Stock standard solutions (1.00 µg/L) - Stock standard solutions can be prepared from pure standard materials or purchased as certified solutions.

6.7.1 Prepare stock standard solutions by accurately weighing about 0.0100 grams of pure material. Dissolve the material in distilled-in-glass quality methylene chloride for analyses performed using column 1 and methanol for analyses performed using column 2. Dilute to volume in a 10-mL volumetric flask. Larger volumes can be used at the convenience of the analyst. If compound purity is certified at 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards can be used at any concentration if they are certified by the manufacturer or by an independent source.

6.7.2 Transfer the stock standard solutions into Teflon-sealed screw-cap bottles. Store at $4^{o}C$ and protect from light. Stock standard solutions should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them.

6.7.3 Stock standard solutions must be replaced after six months or sooner if comparison with check standards indicates a problem.

7. Calibration

7.1 Establish liquid chromatographic operating parameters equivalent to those indicated in Table 1. The liquid chromatographic system can be calibrated using the external standard technique (Section 7.2) or the internal standard technique (Section 7.3).

7.2 External standard calibration procedure:

7.2.1 For each compound of interest, prepare calibration standards at a minimum of three concentration levels by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with 50/50 hexane/methylene chloride for column 1 standards and acetonitrile for column 2 standards. One of the external standards should be at a concentration near, but above, the method detection limit. The other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

7.2.2 Using injections of 5 to 20 µL of each calibration standard, tabulate peak height or area responses against the mass injected. The results can be used to prepare a calibration curve for each compound. Alternatively, the ratio of the response to the mass injected, defined as the calibration factor (CF), can be calculated for each compound at each standard concentration. If the relative standard deviation of the calibration factor is less than 10% over the working range, linearity through the origin can be assumed and the average calibration factor can be used in place of a calibration curve.

7.2.3 The working calibration curve or calibration factor must be verified on each working shift by the measurement of one or more calibration standards. If the response for any

compound varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve or calibration factor must be prepared for that compound.

7.3 Internal standard calibration procedure. To use this approach, the analyst must select one or more internal standards similar in analytical behavior to the compounds of interest. The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Due to these limitations, no internal standard applicable to all samples can be suggested.

7.3.1 Prepare calibration standards at a minimum of three concentration levels for each compound of interest by adding volumes of one or more stock standards to a volumetric flask. To each calibration standard, add a known constant amount of one or more internal standards, and dilute to volume with 50/50 hexane/methylene chloride for column 1 standards and acetonitrile for column 2 standards. One of the standards should be at a concentration near, but above, the method detection limit. The other concentrations should correspond to the expected range of concentrations found in real samples, or should define the working range of the detector.

7.3.2 Using injections of 5 to 20 µL of each calibration standard, tabulate the peak height or area responses against the concentration for each compound and internal standard.

Calculate response factors (RF) for each compound as follows:

$$RF = (A_s C_{is})/(A_{is} C_s)$$

where:

A_s = Response for the compound to be measured.

A_{is} = Response for the internal standard.

C_{is} = Concentration of the internal standard in µg/L.

C_s = Concentration of the compound to be measured in µg/L.

If the RF value over the working range is constant, less than 10% relative standard deviation, the RF can be assumed to be invariant and the average RF can be used for calculations. Alternatively, the results can be used to pilot a calibration curve of response ratios, A_s/A_{is} against RF.

7.3.3 The working calibration curve or RF must be verified on each working shift by the measurement of one or more calibration standards. If the response for any compound varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared for that compound.

7.4 Before using any cleanup procedure, the analyst must process a series of calibration standards through the procedure to validate elution patterns and the absence of interferences from the reagents.

8. Quality control

8.1 Each laboratory using this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications to the method are made, the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike and analyze a minimum of 10% of all samples to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each compound to be measured. Using stock standards, prepare a quality control check sample concentrate in methylene chloride 1000 times more concentrated than the selected concentrations.

8.2.2 Using a pipet, add 1.00 mL of the check sample concentrate to each of a minimum of four 1000-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery (R), and the standard deviation of the percent recovery (s), for the results. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using the appropriate data from Table 2, determine the recovery and single operator precision expected for the method, and compare these results to the values measured in Section 8.2.3. If the data are not comparable, the analyst must review potential problem areas and repeat the test.

8.3 The analyst must calculate method performance criteria and define the performance of the laboratory for each spike concentration and parameter being measured.

8.3.1 Calculate upper and lower control limits for method performance as follows:

$$\text{Upper Control Limit (UCL)} = R + 3s$$

$$\text{Lower Control Limit (LCL)} = R - 3s$$

where R and s are calculated as in Section 8.2.3. The UCL and LCL can be used to construct control charts[6] that are useful in observing trends in performance.

8.3.2 The laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as R $\pm$ s. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated with this method. This ability is established as described regularly.[6]

8.4 The laboratory is required to collect in duplicate a portion of their samples to monitor spike recoveries. The frequency of spiked sample analysis must be at least 10% of all samples or one sample per month, whichever is greater. One aliquot of the sample must be spiked and analyzed as described in Section 8.2. If the recovery for a particular compound does not fall within the control limits for method perfomance, the results reported for that compound in all samples processed as part of the same set must be qualified as described in Section 13.3. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Before processing any samples, the analyst should demonstrate though the analysis of a 1-liter aliquot of reagent water that all glassware and reagents interferences are under control. Each time

a set of samples is extracted or there is a change in reagents, a laboratory reagent blank should be processed as a safeguard against laboratory contamination.

8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. When doubt exists over the identification of a peak on the chromatogram, confirmatory techniques such as liquid chromatography with a dissimilar column, must be used. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9. Samples Collection, Preservation, and Handling

9.1 Grab samples must be collected in glass containers. Conventional sampling practices[7] should be followed; however, the bottle must not be prerinsed with sample before collection. Composite samples should be collected in refrigerated glass containers in accordance with the requirements of the program. Automatic sampling equipment must be as free as possible of Tygon and other potential sources of contamination.

9.2 The samples must be iced or refrigerated at $4^{o}C$ from the time of collection until extraction.

9.3 Adjust the pH of the sample to 6 to 8 with 1N sodium hydroxide or 1N sulfuric acid immediately after sampling.

10. Sample Extraction

10.1 Mark the water meniscus on the side of the sample bottle for later determination of sample volume. Pour the entire sample into a 2-liter separatory funnel. Check the pH of the sample with wide range pH paper and adjust to 7 with 1 N sodium hydroxide or 1 N H_2SO_4.

10.2 Add 60 mL of methylene chloride to the sample bottle, seal, and shake 30 seconds to rinse the inner walls. Transfer the solvent to the separatory funnel and extract the sample by shaking the funnel for 2 minutes with periodic venting to release excess pressure. Allow the organic layer to separate from the water phase for a minimum of 10 minutes. If the emulsion interface between layers is more than one third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may include stirring, filtration of the emulsion through glass wool, centrifugation, or other physical methods. Collect the methylne chloride extract in a 250-mL Erlenmeyer flask.

10.3 Add a second 60-mL volume of methylene chloride to the sample bottle and repeat the extraction procedure a second time, collecting the extract. Perform a third extraction in the same manner and collect the extract.

10.4 Assemble a Kuderna-Danish (K-D) concentrator by attaching a 25-mL concentrator tube to a 250-mL evaporative flask. Other concentration devices or techniques may be used in place of the K-D if the requirements of Section 8.2 are met.

10.5 Pour the combined extract through a drying column containing about 10 cm of anhydrous sodium sulfate, and collect the extract in the K-D concentrator. Rinse the Erlenmeyer flask and column with 20 to 30 ml of methylene chloride to complete the quantitative transfer. Once the flask rinse has passed through the drying column, rinse the column with an additional 30 to 40 mL of methylene chloride.

10.6 Add 1 or 2 clean boiling chips to the evaporative flask and attach a three-ball Snyder column. Prewet the Snyder column by adding about 1 mL methylene chloride to the top. Place the K-D apparatus on a hot water bath, 60 to 65^{o}C, so that the concentrator tube is partially immersed in the hot water, and the entire lower rounded surface of the flask is bathed with hot vapor. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 15 to 20 minutes. At the proper rate of distillation, the balls of the column will actively chatter but the chambers will not flood with condensed solvent. When the apparent volume of liquid reaches 1 mL, remove the K-D apparatus and allow it to drain and cool for at least 10 minutes.

10.7 Remove the macro-Snyder column and rinse the flask and its lower joint into the concentrator tube with 1 to 2 mL of methylene chloride. A 5-mL syringe is recommended for this operation. Add 1 or 2 clean boiling chips and attach a two-ball micro-Snyder column to the concentrator tube. Prewet the micro-Snyder column with methylene chloride and concentrate the solvent extract as before. When an apparent volume of 0.5 mL is reached, or the solution stops boiling, remove the K-D apparatus and allow it to drain and cool

for 10 minutes. If analysis is being performed using column 1 or if sample cleanup is required, proceed with Section 10.9. If column 2 is used and no sample cleanup is required, proceed with Section 10.8.

10.8 Add 10 mL of acetonitrile to the concentrator tube along with 1 or 2 clean boiling chips. Attach a two-ball micro-Snyder column to the concentrator tube. Prewet the micro-Snyder column with acetonitrile and concentrate the solvent extract as before. When an apparent volume of 1 mL is reached, remove the K-D apparatus and allow it to drain and cool for 10 minutes. Transfer the liquid to a 10-mL volumetric flask and dilute to the mark with acetonitrile. Mix thoroughly prior to analysis. Proceed with Section 12 using column 2.

10.9 Remove the micro-Snyder column and adjust the volume of the extract to 1.0 mL with methylene chloride. Stopper the concentrator tube and store refrigerated if further processing will not be performed immediately. If the extract is to be stored longer than two days, transfer the extract to a screw-capped vial with a Teflon-lined cap. If the sample extract requires no further cleanup, proceed with the liquid chromatographic analysis in Section 12 using Column 1. If the sample requires cleanup, proceed to Section 11.

10.10 Determine the original sample volume by refilling the sample bottle to the mark and transferring the water to a 1000-mL graduated cylinder. Record the sample volume to the nearest 5 mL.

11. Cleanup and Separation

11.1 Cleanup procedures may not be necessary for a relatively clean sample matrix. The cleanup procedure recommended in this method has been used for the analysis of various clean waters and

industrial effluents. If particular circumstances demand the use of additional cleanup, the analyst must demonstrate that the recovery of each compound of interest is no less than 85%.

11.2 Slurry 10 g of silica gel in 50 mL of acetone to which has been added 600 µL of reagent water. Transfer the slurry to a chromatographic column (silica gel is retained with a plug of glass wool). Wash the column with 100 mL of methylene chloride. Use a column flow rate of 2 to 2.5 mL/min throughout the wash and elution profiles.

11.3 Add the extract from Section 10.9 to the head of the column. Allow the solvent to elute from the column until the silica gel is almost exposed to the air. Elute the column with 50 mL of methylene chloride. Discard this fraction.

11.4 Elute the column with 60 mL of 6% acetone in methylene chloride. Collect this fraction in a K-D apparatus. Concentrate the column fraction to 1 mL as described in Sections 10.6 and 10.7. If column 1 is being used, proceed with Section 11.5. If column 2 is being used, proceed with Section 11.7

11.5 Add 5 mL of hexane to the concentrate along with 1 or 2 clean boiling chips. Attach a three-ball micro-Snyder column to the concentrator tube. Prewet the micro-Snyder column with hexane and concentrate the solvent extract to an apparent volumn of 1 mL. Allow the K-D apparatus to drain and cool for 10 minutes.

11.6 Transfer the liquid to a 10-mL volumetric flask and dilute to the mark with hexane. Mix thoroughly prior to analysis. If the extracts will not be analyzed immediately, they should be

transferred to Teflon sealed screw-cap vials and refrigerated. Proceed with the liquid chromatographic analysis using column 1.

11.7 Add 10 mL of acetonitrile to the concentrate along with 1 or 2 clean boiling chips. Attach a three-ball micro-Snyder column to the concentrator tube. Prewet the micro-Snyder column with acetonitrile and concentrate the solvent extract to an apparent volume of 1 mL. Allow the K-D apparatus to drain and cool for 10 minutes.

11.8 Transfer the liquid to a 10-mL volumetric flask and dilute to the mark with acetonitrile. Mix thoroughly prior to analysis. If the extracts will not be analyzed immediately, they should be transferred to Teflon sealed screw-cap vials and refrigerated. Proceed with the liquid chromatographic analysis using column 2.

12. Liquid Chromatography

12.1 Table 1 summarizes the recommended operating conditions for the liquid chromatograph. Included in this table are estimated retention times and method detection limits that can be achieved by this method. An example of the separations achieved by column 1 and column 2 are shown in Figures 1 and 2. Other columns, chromatographic conditions, or detectors may be used if the requirements of Section 8.2 are met.

12.2 Calibrate the liquid chromatographic system daily as described in Section 7.

12.3 If an internal standard approach is being used, the analyst must not add the internal standard to sample extracts until immediately before injection into the instrument. Mix thoroughly.

12.4 Inject 5 to 20 µL of the sample extract by completely filling the sample valve loop. Record the resulting peak sizes in area or peak height units.

12.5 The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound can be used to calculate a suggested window size; however, the experience of the analyst should weigh heavily in the interpretation of chromatograms.

12.6 If the response for the peak exceeds the working range of the system, dilute the extract and reanalyze.

12.7 If the measurement of the peak response is prevented by the presence of interferences, further cleanup is required.

13. Calculations

13.1 Determine the concentration of individual compounds in the sample.

13.1.1 If the external standard calibration procedure is used, calculate the amount of material injected from the peak response using the calibration curve or calibration factor in Section 7.2.2. The concentration in the sample can be calculated as follows:

$$\text{Concentration, } \mu g/L = \frac{(A)(V_t)}{(V_i)(V_s)}$$

where:

A = Amount of material injected, in nanograms.

V_i = Volume of extract injected in µL.

V_t = Volume of total extract in µL.

V_s = Volume of water extracted in mL.

13.1.2 If the internal standard calibration procedure was used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.3.2 as follows:

$$\text{Concentration, } \mu g/L = \frac{(A_s)(I_s)}{(A_{is})(RF)(V_o)}$$

where:

A_s = Response for the compound to be measured.

A_{is} = Response for the internal standard.

I_s = Amount of internal standard added to each extract extract in µg.

V_o = Volume of water extracted, in liters.

13.2 Report results in micrograms per liter without correction for recovery data. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

13.3 For samples processed as part of a set where the laboratory spiked sample recovery falls outside of the control limits in Section 8.3, data for the affected compounds must be labeled as suspect.

14. Method Performance

14.1 The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is about zero. The MDL concentrations listed in Table 1 were obtained using reagent water.[1] Similar results were achieved using representative wastewaters.

14.2 This method has been tested for linearity of recovery from spiked reagent water and has been demonstrated to be applicable over the concentration range from 10 x MDL to 1000 x MDL.

14.3 In a single laboratory, Battelle Columbus Laboratories, using spiked wastewater samples, the average recoveries presented in Table 2 were obtained. Seven replicates of each of two different wastewaters were spiked and analyzed. The standard deviation of the percent recovery is also included in Table 2.[1]

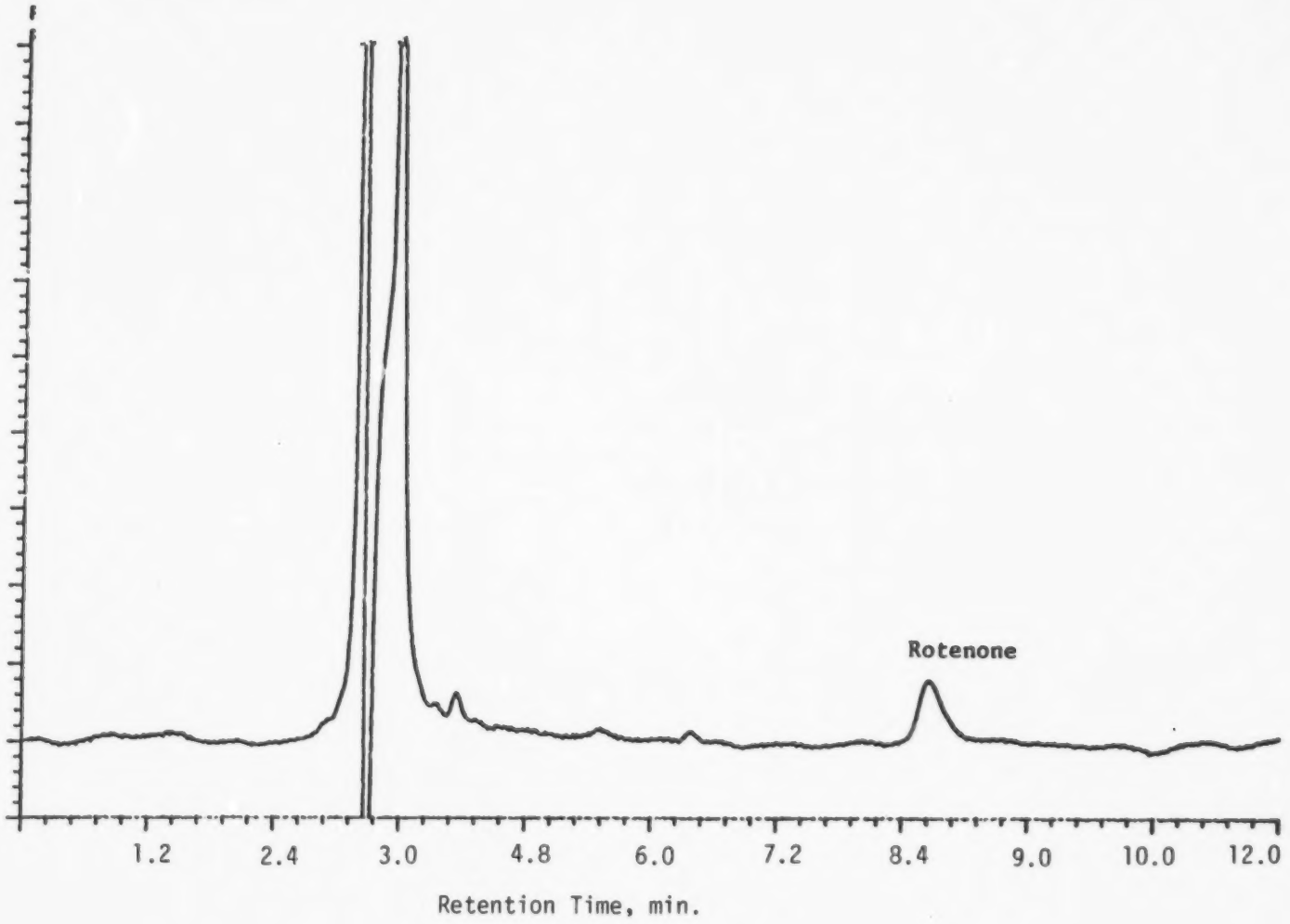

FIGURE 1. HPLC-UV CHROMATOGRAM OF STANDARD SOLUTION REPRESENTING 5 µg/L OF ROTENONE IN WATER (COLUMN 1).

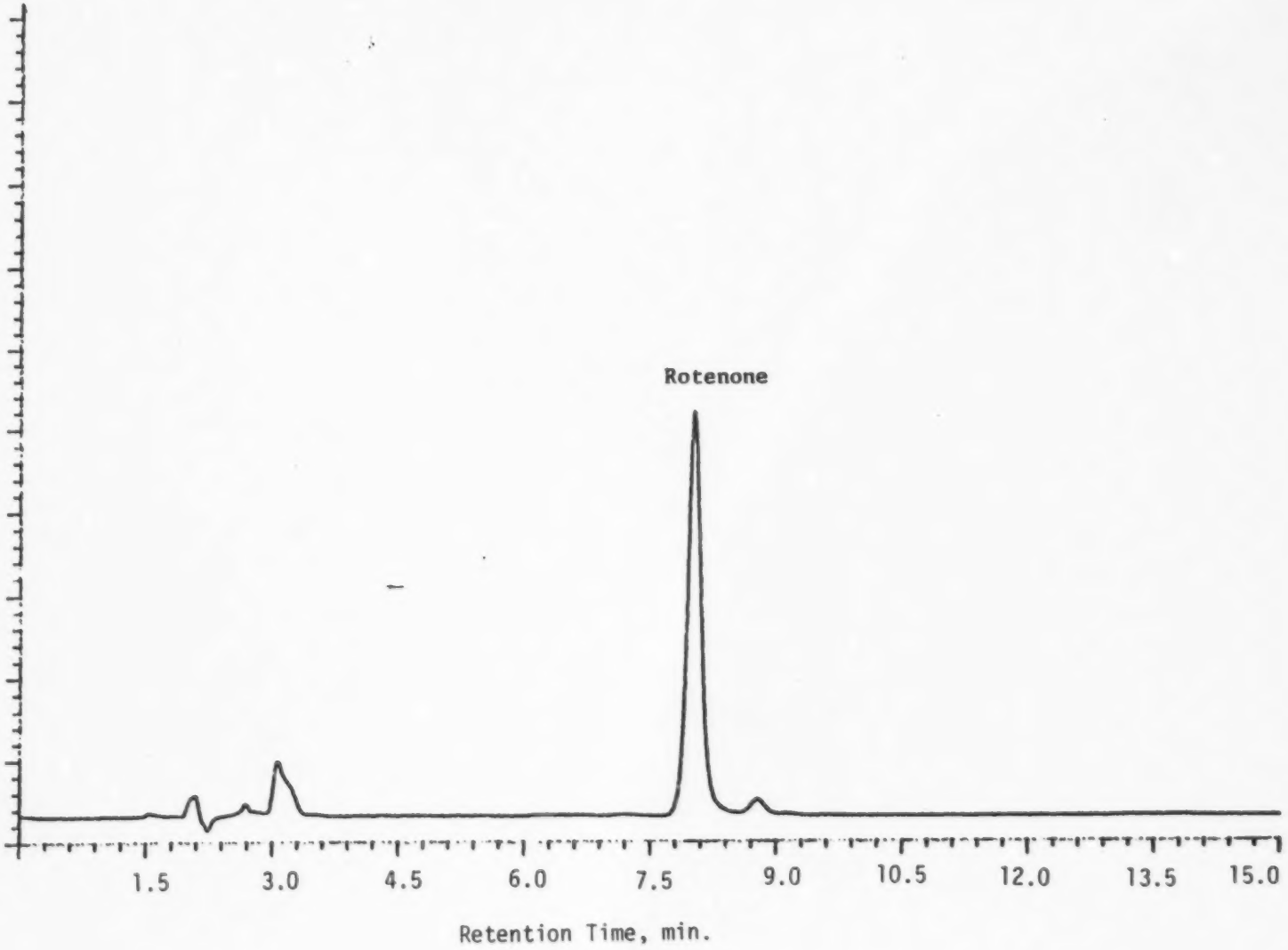

FIGURE 2. HPLC-UV CHROMATOGRAM OF STANDARD SOLUTION REPRESENTING 200 µq/L OF ROTENONE IN WATER (COLUMN 2).

TABLE 1. CHROMATOGRAPHIC CONDITIONS AND METHOD DETECTION LIMITS

Parameter	Retention Time (min.) Column 1	Column 2	Method Detection Limit (µg/L)
Rotenone	8.6	8.0	1.6

Rotenone

Column 1 conditions: Zorbax-CN, 5 micron, 250 x 4.6 mm; 1 mL/min flow; 30/70 methylene chloride/hexane.

Column 2 conditions: Spherisorb-ODS, 5 micron, 250 x 4.6 mm; 1 mL/min flow; 60/40 acetonitrile/water.

TABLE 2. SINGLE LABORATORY ACCURACY AND PRECISION[a]

Parameter	Average Percent Recovery	Standard Deviation, %	Spike Level (µg/L)	Number of Analyses	Matrix Type[b]
Rotenone	85	8	5.5	7	1
	88	3	109	7	1

(a) Column 1 conditions were used.

(b) 1 = pesticide manufacturing wastewater

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